

Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitidis capitata*

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Insect transgenesis is mainly based on the random genomic integration of DNA fragments embedded into non-autonomous transposable elements. Once a random insertion into a specific location of the genome has been identified as particularly useful with respect to transgene expression, the ability to make the insertion homozygous, and lack of fitness costs, it may be advantageous to use that location for further modification. Here we describe an efficient method for the modification of previously inserted transgenes by the use of the site-specific integration system from phage *phiC31* in a tephritid pest species, the Mediterranean fruit fly *Ceratitidis capitata*. First, suitable transgenic strains with randomly integrated *attP* landing sites within transposon-based vectors were identified by molecular and functional characterization. Second, donor plasmids containing an *attB* site, with additional markers, and transposon ends were integrated into *attP* sites by *phiC31* integrase-mediated recombination. Third, transposase-encoding 'jumpstarter' strains were created and mated to transgenic strains resulting in the postintegrational excision of transposon ends, which left stably integrated transgene insertions that could not be remobilized. This three-step integration and stabilization system will allow the combination of several transgene-encoded advantageous traits at evaluated genomic positions to generate optimized strains for pest control that minimize environmental concerns.

insect pest management | *phiC31* integrase | transgene stability

The development of techniques for the genetic manipulation of insect genomes using transposable elements has a strong impact on our understanding of a wide range of biological processes (1). Moreover, insect transgenesis provides powerful tools that have the potential to improve current pest management strategies (2). For a number of economically or medically important species, transgenesis is nowadays exploited to develop strains with different features which may improve the efficacy of existing biocontrol methods such as the sterile insect technique (SIT) (3–7). The SIT is a powerful pest control strategy involving mass rearing of the target pest insect, sterilization, and release of males over the target area. These compete with the wild-type males for mating with wild-type females, thereby causing a reduction in the pest population size (8). The efficiency of this method may take great advantage from the availability of transgenic modifications conferring different useful features such as fluorescent marking (5, 7, 9), transgenic sexing (3), dominant lethality (4, 10), or reproductive sterility (6, 11). Such transgenes are routinely inserted into the genome by the use of transposons as gene vectors, which integrate randomly. This often leads to position effects, which impact on the transgene functionality (6), and/or disrupts gene structure due to insertional mutagenesis, which can cause recessive lethality (12, 13). These phenomena often negatively impact the overall fitness and reliability of the transgenic strain (14). However, once a fit and functional transgenic strain has been generated and characterized, it would be desirable

to take advantage of such an innocuous genomic integration site to manipulate or replace the existing transgene and to introduce additional transgenes to the same genomic position. For this purpose, a site-specific integration system can be used, of which some components need to be already integrated with the initial transposition event. A proven method for generating efficient, unidirectional, and stable integrations is the *phiC31*-mediated site-specific integration system (15). This approach was successfully used to generate *Drosophila melanogaster* transgenic strains (16) and to stably integrate DNA fragments larger than 100 kb at specific attachment *P* (*attP*) sites into the genome of this model species (17). Moreover, the *phiC31*-mediated integration system has been shown functional in a vector-disease species, the yellow fever mosquito *Aedes aegypti* (18).

Also for the economically important insect pest, the Mediterranean fruit fly (medfly) *Ceratitidis capitata* (Diptera: Tephritidae), site-specific recombination will be a valuable tool for the optimization of the currently available and characterized transgenic strains. In this study, we describe a combined approach to manipulate and stabilize medfly transgenic strains, using two strategies in a tephritid species: (i) the *phiC31*-mediated integration system to site-specifically modify previously integrated transgenes; and (ii) *piggyBac*-transposase expressing medfly 'jumpstarter' strains as a tool for efficient remobilization of transposon vectors. This will allow the generation of integrations of a transgene by simple crossings or the excision of inverted terminal repeats (ITRs) leading to the stabilization of specific transgene insertions. Due to the design of the integrating constructs, additional transposable ITRs can be added to the ones introduced by the initial transposon-mediated germline transformation. Adding such ITRs will allow subsequent directional deletion of specific transgene segments, generating, for example, 5' ITR-free strains. After such a stabilization process, the resulting strains should be improved in terms of potential ecological concerns related to the release of transgenic insects in biological control programs. Genetically stable strains have key importance for field applications such as SIT programs, in which strains have to fulfill additional qualifications regarding transgene stability in mass rearing and release compared to strains used in small populations for research. In addition, the site-specific integration will permit the addition of further functional transgenes

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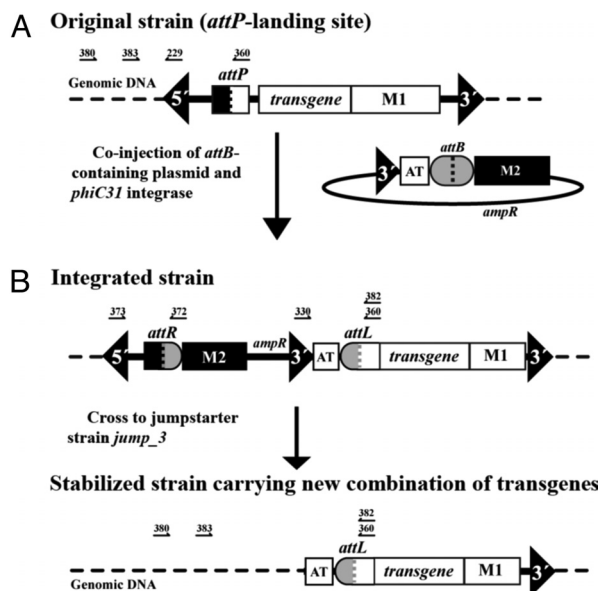


Fig. 1. Three-step strategy for transgene stabilization by site-specific integration and subsequent removal of *piggyBac* ITRs. The original transposon-mediated randomly integrated insertion of a transgene cassette carries one pair of *piggyBac* ITRs (5' and 3'), an *attP* site, the transgene of interest and one fluorescent marker (M1). (A) By co-injection of *phiC31* integrase mRNA and a plasmid containing a 3' *piggyBac* ITR, an *attB* site, an additional transgene (AT) as well as a different fluorescent marker (M2), the plasmid is site-specifically integrated at the *attP* landing site. The recombined insertion then carries one additional 3' *piggyBac* end, two fluorescent markers, the two transgenes, the plasmid backbone (*ampR*), as well as the hybrid sites *attR* and *attL*. (B) The exposure to a transposase source encoded by a jumpstarter strain excises the *piggyBac* cassette including 5' end, *attR*, M2, plasmid backbone, and 3' end. This generates a 5' end-free combination of transgenes.

at a preevaluated genomic position, allowing the development of composite transgenic individuals able to express several advantageous traits.

Results

The transgene modification system described here is based on the combination of two technologies for medfly: (i) A method for site-specific integration via co-injection of capped mRNA integrase from phage *phiC31* and *attB*-containing DNA plasmids into transgenic embryos carrying an *attP* landing site in the genome. (ii) The use of medfly jumpstarter strains that can provide a transposase source by interbreeding, resulting in remobilization or stabilization of recombined transgenic insertions by ITR-mediated excision (Fig. 1).

Development and Test of *piggyBac* Jumpstarter Strains in Medfly. To test medfly *piggyBac* jumpstarter strains as a transposase source for the remobilization or excision of ITRs, we generated the three jumpstarter strains *jump_1*, *jump_3*, and *jump_4* by transformation with a *Minos*-based vector (AH370) containing an *hsp70*-regulated *piggyBac* transposase gene and the medfly *we*⁺ marker. Medfly transformants were generated with this construct in the *we,wp* (19) host strain and selected by red eye pigmentation, with resulting G1 progeny inbred to generate homozygous transgenic progeny. By crossing and inbreeding the strains *sl1-tTA.F1m1* or *sl1-tTA.M6m1* (both marked with *PUBDsRed*; 3) with strain *we,wp*, two jumpstarter-tester strains were obtained that carry the red fluorescent marker *DsRed* homozygously under control of the *D. melanogaster* polyubiquitin promoter (*PUB*) (5, 7, 9) in a white eye background. Subsequently, the three jumpstarter strains were independently crossed in four repetitions to the two tester strains *sl1-*

tTA.F1m1,we,wp and *sl1-tTA.M6m1,we,wp*. Progeny from all 24 crossings were independently backcrossed to *we,wp* and their progeny screened for new patterns of red fluorescence (Fig. 2).

All jumpstarter strains successfully remobilized *piggyBac*-mediated insertions with a similar efficiency as indicated by new expression patterns of the *PUB*-*DsRed* marker. Between 10–20% of all screened progeny showed between one and eight new patterns in respect to the original one. As *jump_1* did not create as clearly identifiable new patterns as *jump_3* and *jump_4* (*jump_3* created e.g., fluorescently marked eyes) (Fig. 2, C8), *jump_3* was selected for the remobilization-stabilization experiments described below.

***attP* Landing Sites and *attB* Integration Constructs.** Site-specific integration was tested at *attP* landing sites from two molecularly, as well as functionally, well characterized and critically evaluated transgenic strains generated by *piggyBac*-mediated germline transformation: (i) the strain carrying the effector gene insertion *TREhs43-hid^{Ala-5}.F1m2* was used to establish a functional embryonic lethality system to generate reproductive sterility. One of the resulting lethality lines, #67, was shown to cause 100% conditional embryonic lethality and males of this strain were highly competitive relative to wild-type medfly males in laboratory and field cage tests (6); (ii) the strain carrying insertion 1260.F-3.m-1 has been evaluated to potentially improve monitoring of pest management programs by fluorescent sperm marking and showed no disadvantages in preliminary laboratory competitiveness assays (7). Both (i) and (ii) transgene insertions are single transposon integrations as proven by Southern blots and inverse PCR (6, 7) and therefore contain a single 52-bp *attP* recombination site that can serve as the landing site for *attB*-containing plasmids (20).

The strain *TREhs43-hid^{Ala-5}.F1m2* carries an EGFP marker driven by *PUB* (6), leading to EGFP expression in the adult thorax (Fig. 3). The strain 1260.F-3.m-1 carries two different markers: turboGFP driven by the *C. capitata* $\beta 2$ tubulin promoter and *DsRed* driven by *PUB*, leading to tGFP expression in the testes and *DsRed* expression in the adult thorax, respectively (2) (Fig. 3).

Since critical evaluation of the strains *TREhs43-hid^{Ala-5}.F1m2* and 1260.F-3.m-1 indicated that their integration sites were particularly useful with respect to transgene expression, the ability to make the insertion homozygous, and the lack of fitness costs (2, 6), they were used in this study to test whether their insertions could be further modified by the use of the *phiC31* integration system. For integrase-mediated germline transformation two plasmids containing a 51-bp *attB* recombination site (20) and an additional 3' *piggyBac* ITR were generated: pSLaf.3'pBac-*attB*-*PUB*-*DsRed*_{af} (#1252) and pSLaf.3'pBac->*attB*-*PUB*-EGFP_{af} (#1255) carrying a red fluorescent (*DsRed*) or a green fluorescent marker (EGFP) under the control of the *PUB* promoter (21, 22), respectively (Fig. 3). When designing these plasmids, we paid particular attention to the orientation and arrangement of the different components: the specific placement of the plasmid backbone including its antibiotic resistance gene between the *attB* and the 3' *piggyBac* ITR makes it possible that this part is removed again by the stabilizing excision (Figs. 1 and 3).

Site-Specific Integration into *attP* Landing Sites. When we performed site-specific integration experiments into the *attP* landing sites of the strains *TREhs43-hid^{Ala-5}.F1m2* (6) and 1260.F-3.m-1 (7), we tested for the highest integration frequencies by injecting different ratios of *attB*-containing plasmid DNA and capped integrase mRNA (23). Four different co-precipitations (DNA/capped-mRNA in ng/ μ L: (i) 200/100, (ii) 200/300, (iii) 200/600, and (iv) 500/400) were prepared for each *attB*-containing plasmid (#1252 and #1255). The co-precipitations of plasmid #1252 were then independently injected into 200 homozygous medfly embryos of the strain *TREhs43-hid^{Ala-5}.F1m2*, resulting in 26, 49, 52, and 65 hatched larvae of which 16, 34, 38, and 39 survived to adulthood, respectively. The four different co-precipitations of plasmid #1255

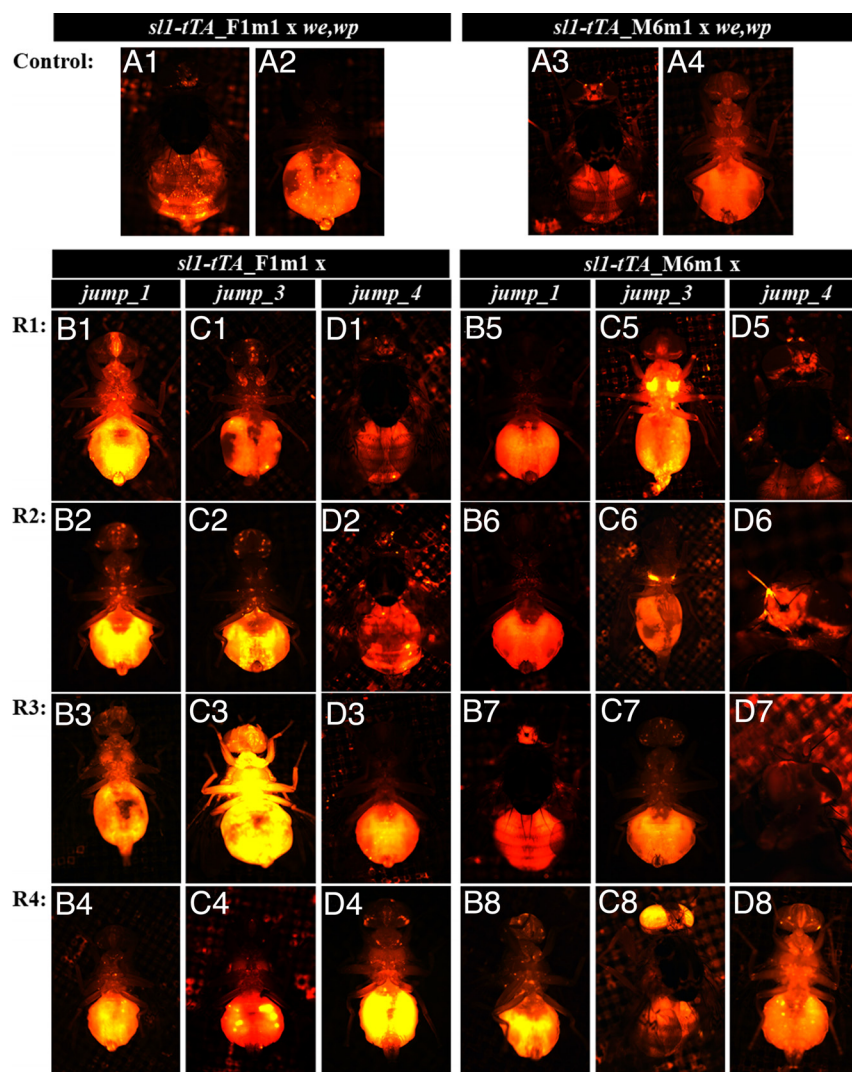


Fig. 2. Activity of *piggyBac* jumpstarter strains. A male from the homozygous medfly strains *sl1-tTA.F1m1.we,wp* or *sl1-tTA.M6m1.we,wp* (in both lines the transgenes are marked with *PUBDsRed*) was mated in four repetitions (R1–R4) to four females of the three independent homozygous jumpstarter strains *jump_1* (B1–B8), *jump_3* (C1–C8), or *jump_4* (D1–D8), respectively (jumpstarter transgenes are marked with *we⁺*). G1 males were outcrossed to *we,wp* females. Their progeny was scored for successful remobilization events, indicated by red fluorescent patterns different from those of the heterozygous control adults from *sl1-tTA.F1m1 x we,wp* (A1 and A2) and *sl1-tTA.M6m1 x we,wp* (A3–A4) (dorsal view: A1 and A3; ventral view: A2 and A4). Flies were observed with the *DsRedwide* filter.

were independently injected into 250 medfly embryos from the strain 1260.F-3.m-1, resulting in 120, 107, 104, and 93 hatched larvae of which 77, 64, 63, and 61 survived to adulthood, respectively. Adults obtained after injections were sorted by sex and backcrossed to wild-type flies in two independent crossings and their offspring were screened for fluorescence expression patterns. In both injection series, transgene integrations were obtained from injections at ratio (iii), 200 ng/ μ L DNA to 600 ng/ μ L capped mRNA, which therefore seems to be the most reliable ratio for successful *phiC31*-mediated site-specific integration in medfly. Ten G₁ flies from injections of plasmid #1252 into *TREhs43-hid^{Ala-5}.F1m2* embryos showed both the original green and the integrated red fluorescence in the body. All 10 flies were from one of the two crossings. One G₁ fly from injections of plasmid #1255 into 1260.F-3.m-1 embryos showed the green fluorescence in the testes, original red fluorescence in the thorax, and newly integrated green fluorescence also in the thorax (Fig. 3).

Initially, site-specific integrations were identified by the phenotypic expression of the respective fluorescent markers. Since *PUB*-mediated fluorescence can vary significantly dependent on the particular genomic insertion site (Fig. 2), the identity of fluorescent patterns of the previously integrated transformation marker (EGFP or *DsRed*) and the new integration marker (*DsRed* or EGFP, respectively) indicates close proximity in the genome which suggests

site-specific integration. For both integration targets, transformed individuals showed the integrated fluorescent markers in a similar pattern as the original transformation marker (Fig. 3). The stable *DsRed* marker (5, 7, 9) showed additional fluorescent expression in the adult, because the EGFP marker loses its intensity in the abdomen during the first hours after eclosion. The two integrated strains, *int.TREhs43-hid^{Ala-5}.F1m2* and *int.1260.F-3.m-1*, were then independently made homozygous by inbreeding recombinant adult flies and screening for homozygous progeny by fluorescence intensity. Since we did not detect any different fluorescent patterns upon integration, we have no evidence for non-specific off-target integrations.

Molecular Characterization of Site-Specific Integration Events. To molecularly confirm the site-specific integrations of *attB*-containing plasmids, strains *int.TREhs43-hid^{Ala-5}.F1m2* and *int.1260.F-3.m-1* were characterized by PCR and DNA sequencing. Fragments covering the *attR* site were amplified from *int.TREhs43-hid^{Ala-5}.F1m2* and *int.1260.F-3.m-1* (196- and 560-bp predicted fragment sizes, respectively) by using the primer pair *mfs-372/mfs-373*. Fragments encompassing the *attL* site were amplified from *int.TREhs43-hid^{Ala-5}.F1m2* and *int.1260.F-3.m-1* (185- and 535-bp predicted fragment sizes, respectively) by using the primer pair *mfs-330/mfs-360* (Fig. 1). All DNA fragments were cloned and sequencing confirmed the

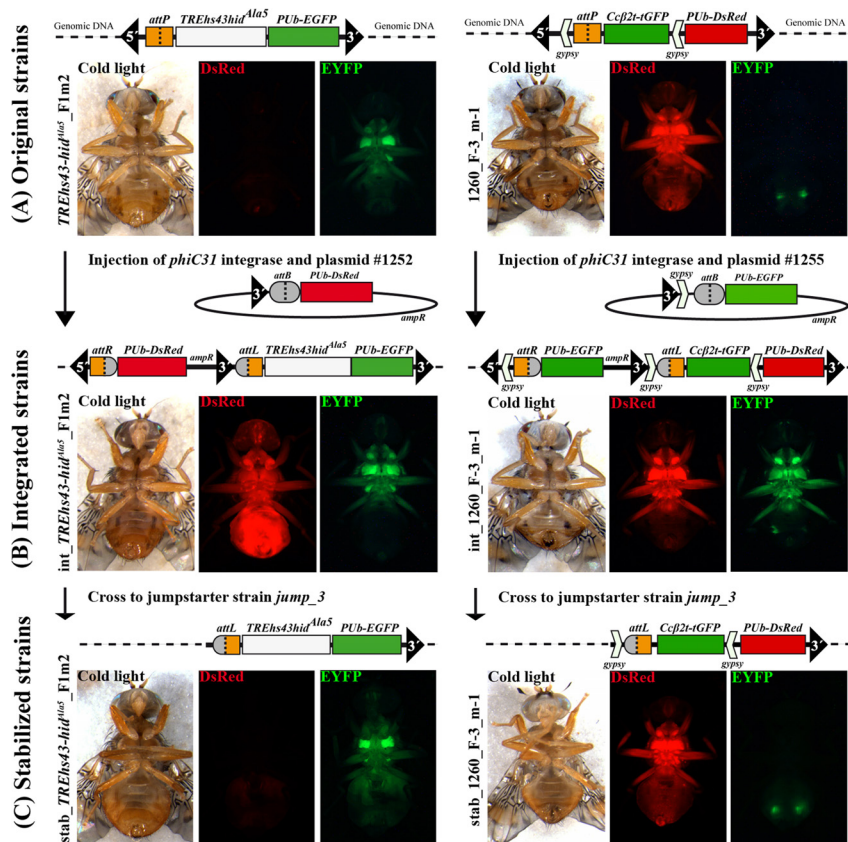


Fig. 3. Stabilization procedure. The original genomic (A), the integrated (B) and the stabilized situation (C) together with the fluorescence marker expression of an adult male from each strain (ventral view) are shown. All flies were observed under cold light and the filter sets DsRed and EYFP, as indicated for each picture. *attB*-containing plasmids were integrated by site-specific recombination at the *attP* landing sites of the original strains *TREhs43-hid^{Ala5}-F1m2* and *1260.F-3.m-1* (A), generating the integrated strains *int.TREhs43-hid^{Ala5}-F1m2* and *int.1260.F-3.m-1* (B). By crossing adults from (B) to the jumpstarter strain *jump_3*, crossing their progeny to *we,wp* and screening by fluorescence microscopy, we developed the stabilized strains *stab.TREhs43-hid^{Ala5}-F1m2* and *stab.1260.F-3.m-1* (C).

successful integration of the *attB*-containing plasmids into the *attP* site of the medfly strains (Dataset S1 and Dataset S2). Integration-generated *attR* and *attL* sites were identical to the respective sequences generated after integration in *Drosophila* (16).

Excision of *piggyBac* ITRs and Stabilization of Transposon-Vector Insertions. As the *attB*-containing plasmids were designed to carry an additional 3' *piggyBac* ITR, their integration into the *attP* site generated a recombinant genomic transgene with three *piggyBac* ITRs, as shown in Figs. 1 and 3. Remobilization of the 5' with either one of the 3' *piggyBac* ITRs, duplicated in tandem, should therefore be possible. As described previously (24), remobilization with the internal 3' ITR sequence should result in stabilization of the remaining 3' ITR and proximal vector sequences. To assay for this, the homozygous jumpstarter strain *jump_3* was crossed separately to the homozygous strains *int.TREhs43-hid^{Ala5}-F1m2* and *int.1260.F-3.m-1*. Progeny were sexed and outcrossed to the *we,wp* strain to identify jumpstarter-free individuals by loss of eye pigmentation (see Fig. S1 for details on the crossing scheme). Excision or transposition events caused by the transposase source were detected in the *G*₂ progeny by loss or change of fluorescent patterns. In the *G*₂ progeny of the cross (i) *int.TREhs43-hid^{Ala5}-F1m2* × *jump_3*, we identified 3% of flies showing only the green, but not red fluorescent thorax/legs (Fig. 3). Five percent of the *G*₂ progeny obtained from the cross (ii) *int.1260.F-3.m-1* × *jump_3* showed red, but not green fluorescent thorax/legs (Fig. 3). This indicated that these flies lost the integrated fluorescent markers by successful excision of part of the integrated cassette including the plasmid backbone flanked by the 5' and a 3' *piggyBac* end, but retained the original transformation marker and, expectedly, also the transgene(s) (Figs. 1 and 3). Independently observed loss and/or change of fluorescent patterns indicate the occurrence of complete excision as well as several combinations of transposition events, which vary

widely due to the presence of three *piggyBac* ITRs in the integrated strains (see Fig. S2).

From crosses (i) and (ii), one *G*₂ white eyed fly verified for the loss of the integrated marker was outcrossed to *we,wp*, their progeny screened again for individuals with the correct fluorescent pattern and white eyes, indicating the absence of the transposase source, and finally inbred. By this procedure we generated the homozygous stabilized strains *stab.TREhs43-hid^{Ala5}-F1m2* and *stab.1260.F-3.m-1* (Fig. 3).

Molecular Characterization of Stabilized Medfly Strains. To molecularly verify the controlled excision of part of the site-specifically integrated DNA construct, PCRs with a forward primer in the flanking genomic region and a reverse primer within the transgene were used. Regions including genomic DNA down to the *attL* site were amplified from *stab.TREhs43-hid^{Ala5}-F1m2* and *stab.1260.F-3.m-1* (460- and 708-bp fragment sizes, respectively) by using the primer pair *mfs-383/mfs-360* and *mfs-380/mfs-382*, respectively (Fig. 1). Both fragments were subcloned and sequencing confirmed the successful and precise *piggyBac* excision of the integration marker and ITRs, leaving the transgene and original transformation marker intact in its original genomic position (Dataset S1 and Dataset S2). In addition, we performed a Southern blot on genomic DNA of the original, integrated, and stabilized strains that further indicates the correct site-specific integration and precise *piggyBac* excision (see Fig. S3). Moreover, this Southern blot also indicates that there are no additional non-specific off-target integrations in the genome of the stabilized lines.

Stability of 5' *piggyBac* ITR-Free Strains. To assay for the stability of the remaining part of the transgene insertions after controlled ITR-excision, we separately crossed the strains *stab.TREhs43-hid^{Ala5}-F1m2* and *stab.1260.F-3.m-1* to *jump_3*. As a control, their respective original versions (*TREhs43-hid^{Ala5}-F1m2* and *1260.F-*

Table 1. Verification of transgene stability

Strains		TREhs43-hid ^{Ala5} _F1m2 (C)			stab.TREhs43-hid ^{Ala5} _F1m2			1260_F-3.m-1 (C)			stab.1260_F-3.m-1		
	Replication	I	II	III	I	II	III	I	II	III	I	II	III
pattern	WT flies	149	160	172	6206	6168	6200	245	186	146	6176	6198	6074
	Original	43	36	31	6130	6204	6150	44	71	34	6128	6096	6130
	New (different)	18 (2)	9 (3)	9 (3)	0	0	0	36 (6)	26 (4)	19 (3)	0	0	0
	total n		627			37058			807			36802	

The numbers of screened G₂ progeny from the strains *TREhs43-hid^{Ala5}_F1m2*, *stab.TREhs43-hid^{Ala5}_F1m2*, *1260_F-3.m-1*, and *stab.1260_F-3.m-1* after crossing them to *jump_3* and outcross progeny to *we,wp* are shown. Numbers of transgenic and non-transgenic (WT) progeny were recorded. All fluorescent expression patterns different (New) from the initial pattern (Original) generated after the crossing to *jump_3* represent a remobilization event which occurred in the parental adult. The number of new different patterns is given in brackets. Original strains used as remobilization controls are marked with (C). Note that the number of fluorescent progeny is less than 50% in the non-stabilized strains and about 50% in the stabilized strains, which indicates a lack of excision in the stabilized strains.

3.m-1) were also crossed to *jump_3*. The progeny of these four crossings were then mated to the *we,wp* strain and their offspring separately scored according to the fluorescence expression patterns. No new fluorescent pattern was detected in three independent repetitions with strain *stab.TREhs43-hid^{Ala5}_F1m2* out of a total of 37,058 progeny or with strain *stab.1260_F-3.m-1* out of a total of 36,802 progeny (Table 1). On the contrary, the control with the original strains crossed to *jump_3* always generated new fluorescent patterns (in total 36 progeny out of 627 for *TREhs43-hid^{Ala5}_F1m2* and 81 progeny out of 807 for *1260_F-3.m-1*). Moreover, whereas in the control less than 50% of the progeny showed fluorescence due to excision events, in the stabilized strains 50% of the progeny remained fluorescent which suggests that no excision events took place. This indicates that the presence of only one ITR (3' *piggyBac* end) (Figs. 1 and 3) in the stabilized strains does not mediate excisions or transpositions in *C. capitata* similar to what has been shown in *D. melanogaster* (24). Furthermore, when propagating the homozygous stabilized strains over many generations, we have no indication of transgene loss. These results support the conclusion that the transgenic insertions were successfully stabilized and were no longer a substrate for further transposase-mediated remobilization events.

Discussion

The established *piggyBac* jumpstarter strains can be used to generate new integrations of transgenes without the need for re-injecting vector and helper plasmids to perform germline transformation. Since position effects can greatly affect the usability of transgene insertions (3), the remobilization of transgenes by jumpstarter strains enables the quick generation of many new genomic insertions that then can be tested for transgene effectiveness and the ability to become homozygous as well as potential fitness costs.

The successful application of the *phiC31*-mediated site-specific integration system will confer great flexibility to medfly transformation technology by: (i) enabling the combination of different transgenic systems in this pest species at preevaluated genomic positions; (ii) facilitating the stabilization of transgenic insertions; and (iii) potentially permitting the integration of large DNA fragments as it has been reported for *D. melanogaster* (17). Using site-specific systems, researchers should be able to create transposon-free cassette exchange systems for replacement of original transgenes with any desired transgenic construct and to more efficiently combine different systems. This will enable the creation of transgenic strains carrying diverse transgenes in tandem (Fig. 1), which will be inherited by their progeny together. For example, a combination of a conditional embryonic lethality (6), a sperm marking (7), and a sexing system (3, 25) could be put into effect in medfly with the described strains. Deleting *piggyBac* ends could then further increase the stability of such strains. Having all these systems in one genomic position, proven to be non-compromising and effective, should improve the sterilization, sexing and monitoring processes of SIT programs. We would expect that the

generated stabilized lines show the same fitness as their predecessors, since the stabilization procedure only removes part of the transposon construct that has not been there in the wild-type situation. Nevertheless, the fitness of the stabilized lines needs to be verified by large scale field cage competition assays before an eventual field application.

The presence of *attP* landing sites in the genome of already characterized transgenic strains will enable the insertion of different transgenes at precise positions for comparative analysis. This advance should greatly facilitate the detailed study of medfly regulatory elements such as enhancers, silencers, and insulators or the function of medfly protein variants by the use of expression systems (26).

Transgenic technology relying on transposon-based vectors has proven to be a powerful tool for the genetic manipulation of insect genomes, which can be tested and applied to the improvement of current environment-friendly pest control methods (1). However, a fundamental requirement for a safe release of genetically modified insects into the wild is the development of systems inert to any potential mobilizing transposase source present in the environment. Therefore the development of non-autonomous transposons whose ends can be deleted after integration to cause effective immobilization of the inserted transgene is essential to ultimately taking transgenic improvements of pest management programs from the lab to the field (27). Here we demonstrate that it is possible to generate 5' *piggyBac* ITR-free insertions in a major pest species and that these modified insertions are inert to the *piggyBac* transposase, which might be inadvertently present in the transgenic host strain. This represents an important step forward toward the safe field use of transgenic insects.

Methods

Medfly Strains. The medfly strains *we,wp* (phenotype white eyes, white pupae) and *Egll* (WT) were received from Gerald Franz (IAEA). Both WT and transgenic medfly strains were reared under standard laboratory conditions (28).

Plasmid Construction. Each of the *pBac*-jumpstarter strains contains the pMi{Cwhite+; hspBac} (AH.370) generated by an exchange of the 3×P3-DsRed from pMi{3xP3-DsRed; hsp70-piggyBac} (29) for the medfly *white* gene (30). The *attB*-containing constructs *pSLaf.3'pBac-attB.Pub-DsRed.af* (#1252) and *pSLaf.3'pBac->attB.Pub-EGFP.af* (#1255) were generated by ligating the *Bgl*II/*Afl*III cut fragment *Pub-DsRed* (3.0 kb) and *Pub-EGFP* (3.2 kb) from #1200 and #1201 (7), in the *Bgl*II/*Afl*III cut vectors *pSLaf.3'pBac-attB.af* (#1250) and *pSLaf.3'pBac->attB.af* (#1251), respectively. We created #1250 or #1251 by cloning hybridized primers *mfs-205* (GATCCTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCGGGGCGTACTCCACTCACAA)/*mfs-206* (GATCTGTGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCAGCCCTGGCACCCGAG) in the *Bam*HI/*Bgl*II site of *pSLaf.3'pBac.af* or *pSLaf.3'pBac->.af*, respectively. The *pSLaf.3'pBac->.af* plasmid was generated by ligating a 0.4-kb gypsy element, amplified by PCR on *pBac{3xP3-DsRed>af}* (31) with the primer pair *mfs-197* (CAGTGGGCCCGGTACCTATTGCGCAAAAC)/*mfs-198* (ACTCGGATCCGGCTAAATGGTATGGCAAG) and subsequent digestion with restriction enzymes *Apal*/*Bam*HI, in the *Apal*/*Bam*HI cut *pSLaf.3'pBac.af*. To generate *pSLaf.3'pBac.af*, a 1.3-kb 3' *pBac EcoRV*/*Hpa*I fragment from p3E1.2 was ligated into the *Sma*I cut *pSLaf.1180fa* (32).

Medfly Germline Transformation. Jumpstarter Strains. Medfly transformation using *Minos* transposable elements marked by *hscCw* (33) to generate *piggyBac* jumpstarter strains was performed using standard methods (34) into the *we, wp* strain.

Site-Specific Integration. The capped *phiC31* integrase mRNA was transcribed from the *Bam*HI cut plasmid *pcDNA3.1-phiC31* (23) according to the protocol of the mMESSAGE mMACHINE T7 kit (Ambion). *phiC31* capped integrase mRNA was co-precipitated with *attB* plasmids #1252 or #1255, and injected in 200–250 embryos of from the strains *TREhs43-hid^{Ala-5}F1m2* or 1260.F-3.m-1, respectively. All G₀ adults were sexed and mated to 20 WT flies of the opposite sex. The G₁ progeny were screened for the presence of fluorescence by epifluorescence microscopy using the stereomicroscope LEICA MZ 16 FA and Filter sets DsRedwide (Ext. 546/12; Emm. 605/75), DsRed (Ext. 545/30; Emm. 620/60), and EYFP (Ext. 500/20; Emm. 535/30). Recombinant G₁ individuals were bred to homozygosity.

Molecular Characterization. For the molecular characterization, touchdown PCRs were performed (2 min at 94 °C; 6 cycles of 30 s at 94 °C, 45 s at 70 °C (–2 °C each cycle), 1.5 min at 72 °C; 27 cycles of 30 s at 94 °C, 45 s at 56 °C, 1.5 min at 72 °C; and 5 min at 72 °C) on genomic DNA from the original strains *TREhs43-hid^{Ala-5}F1m2* and 1260.F-3.m-1 and from the integrated strains *int.TREhs43-hid^{Ala-5}F1m2* and *int.1260.F-3.m-1* using the BD Advantage 2 PCR Kit (BD Biosciences). To verify the genomic positions of the transgenes in *TREhs43-hid^{Ala-5}F1m2* the primer pair mfs-383 (GTCTGGACTGTAAGGTGGTGATTA)/mfs-360 (GCCTAGCGAC-CCTACGCCCACTGAG) was used, while primer pair mfs-380 (GGCCACATT-TGTCTAACTCT)/mfs-229 (CAGTGACATTACCGATTGACAAGCAGCGCTCAC) was used for 1260.F-3.m-1. To verify the *attR* integration site in the integrated strains the primer pair mfs-373 (ATCTTGACCTTGCCACAGAGGACTATTAGAG)/mfs-372 (TTGAGCTCGAGTCTGTGAGGTGAGTACG) was used. To verify the *attL* integration site of *int.TREhs43-hid^{Ala-5}F1m2* the primer pair mfs-330 (GCT-CATCGACTTGATATTGTCGACAC)/mfs-360 was used, while for *int.1260.F-3.m-1* the primer pair mfs-330/mfs-382 (ATTGACCTACGCCCACTGAG) was used. PCR products were cloned into pCRII vectors (Invitrogen) and sequenced.

Touchdown PCRs (2 min at 94 °C; 5 cycles of 30 s at 94 °C, 45 s at 68 °C (–2 °C each cycle), 1 min at 68 °C; 27 cycles of 30 s at 94 °C, 45 s at 58 °C, 1 min at 68 °C; and 2 min at 68 °C) for the molecular characterization of stabilized strains were performed on genomic DNA from the strains *stab.TREhs43-hid^{Ala-5}F1m2* and *stab.1260.F-3.m-1* using the BD Advantage 2 PCR Kit (BD Biosciences). To verify the remobilization of the integrated markers, the 5' *piggyBac* end and one 3' *piggyBac* end, the primer pair mfs-383/mfs-360 was used for the strain *stab.TREhs43-hid^{Ala-5}F1m2* and mfs-380/mfs-382 for the strain *stab.1260.F-3.m-1*. PCR products were cloned into pCRII vectors (Invitrogen) and sequenced.

piggyBac ITR Excision Assay. Four medfly strains were used to perform the excision assay: *int.TREhs43-hid^{Ala-5}F1m2*, *int.1260.F-3.m-1*, *jump_3*, and *we, wp*. The integrated strains *int.TREhs43-hid^{Ala-5}F1m2* and *int.1260.F-3.m-1* carry both the genetically linked red and green fluorescent markers as homozygous alleles. *jump_3* carries in *we* background the genetically linked medfly *hscCw⁺* marker gene (25) and the *piggyBac* transposase as homozygous alleles. To excise the integration marker together with the transposable ends, the homozygous strains *int.TREhs43-hid^{Ala-5}F1m2* and *int.1260.F-3.m-1* (Fig. 3) were separately crossed to the homozygous jumpstarter strain *jump_3*. From both crossings progeny heterozygous for both markers and the *piggyBac* transposase was outcrossed to *we, wp*. Their progeny was screened for green and red fluorescent expression patterns. White eyes indicated the absence of the genetically linked *hscCw* marker and *piggyBac* transposase gene. Successful excision or remobilization events were identified by the loss or the appearance of new fluorescent expression patterns of the *PUB*-mediated fluorescence, respectively.

Stability Test. To check the stability of the transgenic insertions of *stab.TREhs43-hid^{Ala-5}F1m2* and *stab.1260.F-3.m-1*, 50 males from each of these two strains were crossed to 50 homozygous *jump_3* females in three independent repetitions. Of their progeny, 250 virgin females were then crossed to 250 *we, wp* males. In parallel, at a lower scale, the same experimental design was applied to the two original strains *TREhs43-hid^{Ala-5}F1m2* and 1260.F-3.m-1 as a control: 10 males from each of the original strains were crossed to 10 homozygous *jump_3* females and 10 virgin females of their progeny were then crossed to 10 *we, wp* males. About 12,350 progeny from each independent crossing of stabilized lines and between 200–325 progeny from each independent crossing of the original lines were screened for fluorescence patterns. The numbers of flies showing (i) the WT phenotype, (ii) the original fluorescence pattern, (iii) a different fluorescence pattern and (iv), the number of new patterns generated by the transgene remobilization events were scored.

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